

Polymorphism in the vitamin D receptor gene and bone mass in African-American and white mothers and children: a preliminary report

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Abstract

Objective—To evaluate the contribution of polymorphisms in the vitamin D receptor (VDR) gene to ethnic variations in bone mass in mother and children from different ethnic origins.

Methods—VDR genotypes and bone mass in 43 African-American and white women, mean age 38.2 years, and 41 of their children were studied. All children had a whole body bone mass measurement at age 9, and 39 had follow up measurements at age 11, while all the mothers had a single measurement. DNA was extracted from peripheral blood samples, subjected to polymerase chain reactions using primers specific for the VDR gene, and the *Bsm1* restriction fragment length polymorphism defined.

Results—There was a significant ethnic difference in the VDR genotype frequencies among the adults and the children. No African-American subjects had the genotype "BB". In contrast, there was a 25% frequency of the "BB" genotype in the white adults and 24% in the white children. After pooling the ethnic groups, the mean bone mass in the "bb" genotype was significantly higher than in the "BB" genotype among the mothers, but this was not found in the children at baseline. However, by age 11, those with the "Bb" or "bb" genotypes had a larger gain in bone mass than those with "BB".

Conclusion—These data support the suggestion that the ethnic difference in VDR genotype frequencies, together with the association between the genotypes and bone mass, may help to explain the well known ethnic differences in bone mass. Further, our observations suggest that VDR polymorphism may have an effect on bone mass during puberty as peak bone mass is accumulated.

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such that the "bb" genotype had the highest bone mass, "BB" the lowest, and the heterozygote was intermediate. The twin study portion of this report was subsequently retracted,³ but a genotype effect was shown in the population based sample. The results of numerous subsequent investigations in several different countries have had mixed results,⁴⁻⁹ but a meta-analysis supports the contribution of the VDR genotype to bone density.¹⁰

Investigations in American populations have mainly included white women, though some have included minorities. The data of Hustmyer and Peacock for a small number of black subjects (n=19) and Asians (n=16) suggested that the VDR is highly polymorphic and that allele frequencies vary with ethnicity.¹¹ Findings of more recent studies are variable, and Fleet *et al* reported no significant ethnic difference in the *Bsm1* genotype distribution in young adult white and African-American women.¹² Neither was there significant interaction of ethnicity and genotype on the bone mineral density (BMD) of the femoral neck and lumbar spine in this study, but there was a significant relation between the genotypes and bone density in the group as a whole. Harris *et al*, using the *Fok1* polymorphism, did find differences in its distribution among premenopausal women.¹³ They suggest that this polymorphism influences peak bone density, and that ethnic differences in the genotype frequencies may help to explain some of the ethnic difference in femoral neck BMD. Zmuda *et al* investigated three VDR gene polymorphisms (*Bsm1*, *Apal*, and *Taq1*), bone turnover, and rates of bone loss in older African-American women.¹⁴ They did not find an association between VDR gene polymorphisms and BMD or indices of bone turnover in this group.

Only a few studies have been conducted in children.¹⁵⁻¹⁸ Two of these studies, reported in abstracts,^{15 16} were cross sectional and found an association between VDR polymorphisms and BMD in prepubertal girls. A recent study by Sainz *et al* reported that VDR genotypes predict bone density in prepubertal Mexican-American girls.¹⁷ The only reported longitudinal study found no association between VDR genotypes and bone gain in children or adolescents.¹⁸ To the best of our knowledge, there are no other VDR data for non-white children or for families. In our multiethnic study, which includes longitudinal data on children and one measurement in their mothers, we examined whether allelic variants in the VDR gene are associated with whole body

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In 1992, Morrison *et al* reported that allelic variation in several polymorphisms at the vitamin D receptor (VDR) gene locus could be used to predict bone turnover (specifically, osteocalcin levels),¹ and later reported an association with bone mass in twins, and in a large group of white Australian women.² In this second study, the *Bsm1* restriction fragment length polymorphism (RFLP) genotypes ("BB", "Bb", and "bb") were characterised by significant differences in mean bone density

Table 1 Descriptive statistics of sample group

	White adults (n=24)	African-American adults (n=19)	White children (n=25)	African-American children (n=16)
Age (years)	38.5	37.9	9	9
Height (cm)	164.6	164.4	134.9	139.1
Weight (kg)	70.8	73.6	32.4	37.1
BMI* (kg/m ²)	26.1	27.2	17.7	18.9
BMC* (g)	2332.3	2535.4	1167.8	1290.4
BMD* (g/cm ³)	1.093	1.152	0.828	0.858

*BMI = body mass index; BMC = bone mineral content; BMD = bone mineral density.

No significant differences based on ethnicity in either the adults or children in any of the variables above.

bone mineral content and bone mineral density. We also investigated whether there were ethnic (African-American compared with white) differences in the genotype frequencies and distribution. Our longitudinal study of the accumulation of bone mass in children,¹⁹ including some of their parents who also had bone mass measurements,^{20, 21} provided a unique opportunity for a pilot study to investigate the possible association between the *BsmI* genotypes and bone mass in African-American and white subjects and in two generations.

Methods

SUBJECTS

The children in this pilot study of the VDR gene are a subsample of a cohort of 790 third graders, average age 9, who were participating in a four year longitudinal study of bone mass.¹⁹ They were recruited by contacting the parents of all children enrolled in the third grade in a school district adjacent to Detroit. Of the 1180 children whose parent(s) completed a telephone survey, 790 (67%) came into our research centre for a baseline visit. All children were ambulatory and able to lie still for 10 minutes for the bone mass measurement. Five of the children were excluded from later analyses because of systemic or genetic disorders affecting bone and mineral metabolism. The sex and ethnic distribution of the participants was representative of the community.¹⁹ Although we deliberately excluded invasive procedures from our study plan in order to enhance recruitment and retention, we undertook a pilot study of VDR genotypes and bone mass in a subset of the children and their mothers (n=49). When we began the pilot study, the research coordinator asked every mother-child pair who came in for their study visits whether they would both agree to blood being drawn as a separate and optional substudy. When 50 pairs had agreed to participate, we closed recruitment for the pilot study. We have obtained complete genotype and bone mass data in 43 mothers and 41 of their children. Of these, 39 children had follow up BMD measurements at age 11, while the mothers had a single BMD measurement. The remainder were lost to follow up. Ethnicity was determined by self identification. Of the 43 premenopausal mothers, 19 were African-American and 24 were white. Of the 41 prepubertal children, 16 were African-American and 25 were white. Table 1 gives descriptive statistics for mothers and children. In all cases, children were the mothers' biological offspring, and the ethnicity of the biological father was

the same as that of the mothers and children. Both boys and girls were included because the data from our cohort of nearly 800 children show that there is no significant sex difference in whole body bone mass at this age.¹⁹

BONE MEASUREMENTS

Whole body bone mineral content (BMC) and BMD were evaluated by dual energy x ray absorptiometry (DXA) with a Hologic 1000 W instrument. Published DXA standards were used with a precision of whole body at 1%.²² The mothers had a single bone density measurement, whereas all 41 children had measurements at age 9, and 39 children again at age 11.

DNA ANALYSIS

Peripheral whole blood was drawn from each of the subjects and was frozen at -70°C until DNA extraction. Genomic DNA was isolated using the phenol:chloroform extraction procedure.²³ The method resulted in high molecular weight DNA that was free of RNA contamination. Genotypes of the VDR RFLP, *BsmI*, were determined by polymerase chain reaction (PCR) amplification followed by restriction enzyme digestion of the PCR product. 100 ng of each DNA sample was combined in a reaction mixture with the VDR gene primer pair,² PCR buffer, MgCl₂, and dNTPs (2'-deoxynucleoside 5'-triphosphates) in distilled water, and AmpliTaq DNA polymerase (Perkin Elmer Cetus) was added to a final concentration of 5 U/μl. The reaction was started by heating the mixture at 94°C for four minutes to denature the DNA complex, then annealing the primers at 65°C for 50 seconds, and extending the primer sequence at 72°C for 20 seconds. This cycle was repeated (with a 20 second denaturation phase at 94°C) 30 times using the DNA thermal cycler (Perkin Elmer Model 480). After this amplification 10 μl of each reaction solution was mixed with 1 μl of loading buffer, loaded on a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma, St Louis, MO) and run at 50 V for 30 minutes, confirming the presence of the correct PCR product.

The PCR product was approximately 800 base pairs and contained a fragment of the VDR, which flanks the *BsmI* restriction enzyme cut site. This PCR product was then

Table 2 Vitamin D receptor genotype frequencies within 43 adults grouped by ethnic origin. Results are given as number (%) of patients.

Ethnic group	BB	Bb	bb
African-American	0/19 (0)	10/19 (53)	9/19 (47)
White	6/24 (25)	7/24 (29)	11/24 (46)
Pooled	6/43 (14)	17/43 (40)	20/43 (47)

Table 3 Vitamin D receptor genotype frequencies within 41 children grouped by ethnic origin. Results are shown as number (%) of children

Ethnic group	BB	Bb	bb
African-American	0/16 (0)	8/16 (50)	8/16 (50)
White	6/25 (24)	14/25 (56)	5/25 (20)
Pooled	6/41 (15)	22/41 (54)	13/41 (32)

Table 4 Mean bone mass by genotype for adults and children. Values are shown as mean (SD)

Group	Bone mass variable	BB	Bb	bb
Adults (n=43)	BMC [¶] (g)	1863 (491)*	2472 (477)	2479 (319)
	BMD [¶] (g/cm ²)	0.970 (0.14)†	1.1 (0.11)	1.15 (0.07)
Children aged 9 (n=41)	BMC (g)	1003 (468)‡	973 (284)	926 (233)
	BMD (g/cm ²)	0.80 (0.10)‡	0.79 (0.06)	0.79 (0.05)

*BB different from bb, $p < 0.005$; BB different from Bb, $p < 0.006$, Bb and bb not different, $p > 0.50$.

†BB different from bb, $p < 0.001$; BB different from Bb, $p < 0.01$.

‡Not significant.

¶BMC = bone mineral content; BMD = bone mineral density.

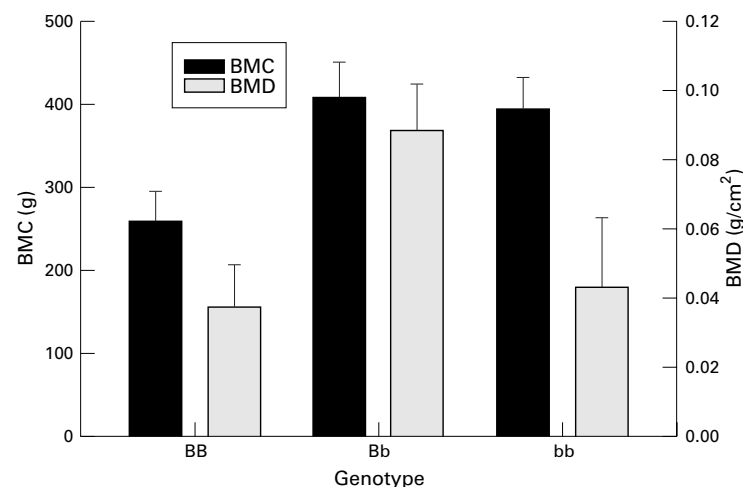


Figure 1 The influence of the VDR genotype on bone mineral content (BMC) and bone mineral density (BMD) on bone mass in adults. Values shown are mean and standard deviation.

digested by the addition of the restriction enzyme *BsmI* (New England Biolabs, Beverly, MA) to a final concentration of 1 U/μl at 65°C for 18 hours. Digested PCR products were separated electrophoretically on a 2% agarose gel containing ethidium bromide. VDR genotypes were determined based upon the fragmentation pattern. Digests were visualised under ultraviolet light and the gel pattern photographed. Homozygotes that lack the *BsmI* site ("BB") exhibited the intact PCR product, with a single band at 800 bp. Homozygotes for the gene containing the *BsmI* site ("bb") had

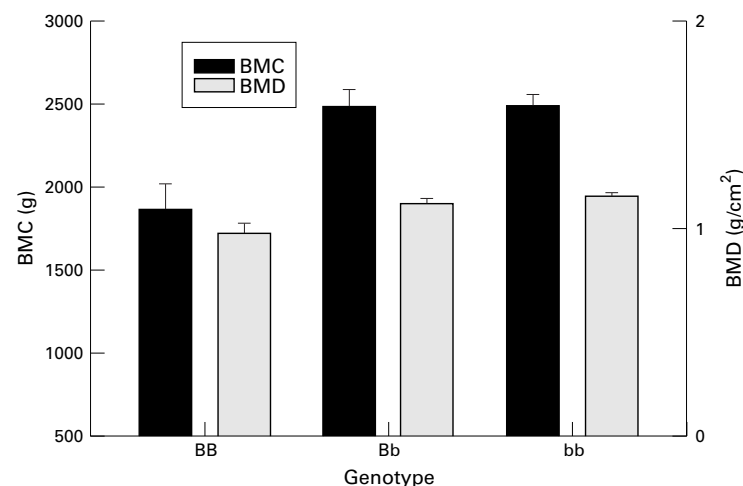


Figure 2 Influence of the VDR genotype on the increase in bone mineral content (BMC) and bone mineral density (BMD) on bone mass in children from age 9 to age 11. Values shown are mean and standard deviation for individual change over the two year interval.

two bands at approximately 650 and 150 bp, which corresponded to the restriction fragments. Patient samples that were heterozygous for the polymorphism ("Bb") had both the intact PCR product (800 bp) and the restriction fragments (650 and 150 bp), with three bands present on the gel.

STATISTICAL ANALYSIS

Statistical analyses were done using SPSS (SPSS Inc, Chicago, IL). To evaluate differences between or among groups, analysis of variance was performed with post hoc pairwise testing, when necessary, using the Scheffé test. Differences among genotype frequencies were evaluated by χ^2 tests, with Yates's correction as necessary. Simple linear regressions were also used. Expected genotype frequencies were estimated from the maternal genotype distribution using the Hardy-Weinberg equilibrium model, and these were compared with observed frequencies in the children using χ^2 analyses. An α level of 0.05 was selected for significance for all the statistical tests.

Results

ETHNIC DISTRIBUTION OF VDR

A significant ethnic difference in the VDR genotype frequencies was found among the adults and the children ($p = 0.01$ and 0.02 , respectively), with the finding that no African-American subjects had the genotype "BB" (tables 2 and 3). In the African-American women and children there was an approximately equal distribution between the "Bb" and the "bb" genotypes. In contrast, there was a 25% frequency of the "BB" genotype in the white adults and 24% in the white children.

Based on the Hardy-Weinberg model, the expected distribution of genotypes in the children (with the ethnic groups combined), predicted from the pooled parental allele frequencies, did not differ significantly from the observed genotype frequencies in the children ($p > 0.10$). The mean (SD) age of the mothers was 38.2 (5.4) years, and there was no significant ethnic difference in mean age ($p > 0.05$).

INFLUENCE OF VDR GENES ON BONE MASS

The examination of the distribution of bone mass across genotypes in the African-American and white subjects separately showed a significant ($p < 0.01$) difference for the white adults, but not for the white children or the African-American adults or children. As the small cell sizes might have masked a real difference, the ethnic groups were pooled to examine the influence of VDR polymorphism on the BMC and BMD regardless of ethnicity (table 4). In the pooled analysis, there was a significant influence of the VDR genotype on bone mass in the adults regardless of ethnicity ($p < 0.001$ for BMD and $p < 0.003$ for BMC, for the model). Post hoc pairwise testing showed that the "Bb" and "bb" genotypes were associated with the highest BMC and BMD, whereas subjects with the lowest BMC and BMD expressed the "BB" genotype ($p \leq 0.01$, fig 1).

There were no statistically significant bone mass differences across genotypes among the children at baseline (age 9) or at age 11. However, there was a trend towards a significant difference ($p=0.08$) in BMD across genotypes (fig 2). A similar trend was seen for BMC but this was not significant ($p>0.10$). The absolute and percentage increases over two years for BMC were as follows: for "BB" 127 g (12.7%); for "Bb" 415 g (43%); and for "bb" 410 g (44%). Finally, a regression analysis of children's and mothers' bone mass Z scores found only a weak association, with an r^2 of 0.09, suggesting that the mothers' bone mass is not a good predictor of their children's prepubertal bone mass.

Discussion

We found that the distribution of the VDR *BsmI* genotypes is different for African-American and white subjects, with the finding that no African-American subjects had the genotype "BB". This finding is in contrast to that of Fleet *et al* who reported no significant ethnic difference in genotype distribution in 83 white and 72 African-American women aged 20–40.¹² In their study, also, no significant interaction of ethnicity and genotype on BMD of the femoral neck and lumbar spine was found.¹² However, there was a significant relation between the genotypes and bone density in the group as a whole, which corroborates our findings. Thus we found that among the adults in our study group there was a higher bone mass associated with the "bb" genotype when the ethnic groups were pooled. Using a different locus, a start codon polymorphism detected with the endonuclease *FokI*, Harris *et al* found differences in its distribution among black or white premenopausal women.¹³ They suggest that this polymorphism seems to influence peak bone density, and that ethnic differences in the *FokI* genotype frequencies may help to explain some of the ethnic difference in femoral neck BMD. The data from our present study support this suggestion for the *BsmI* polymorphism as well. Although this association was not apparent in children at age 9, there is a trend toward the adult pattern among the children at age 11, just before puberty is expected to begin. Possibly, the "BB" variant has the slowest rate of bone density increase, resulting in the lowest bone mass in adulthood. Conversely, the subjects whose genotypes contain the "b" allele may increase their bone mass at a greater rate, thereby resulting in adults with the highest bone mass. These observations may imply an increasing effect of VDR on bone mass during puberty as peak bone mass is accumulated, though Gunnes *et al* did not find evidence for this in their study of boys and girls in Norway.¹⁸

One limitation of our study is that the sample sizes and genotype distributions resulted in small sample sizes for some of the comparisons across genotypes. However, when examining data from three other studies that included African-American subjects,^{11 12 14} the frequency of "BB" ranges from 0% to 13%. The average

frequency in those three studies and ours is 8.7% ($n=197$). Thus the frequency of this genotype is low, but it is not absent, in the African-American gene pool. In our study the low sample size limited our ability to evaluate the ethnic groups separately. Therefore, our results relate to the association of VDR genotypes and bone mass regardless of ethnicity. In fact, these data may suggest that some of the ethnic difference in bone mass is due to an ethnic difference in the distribution of genotypes. There is no a priori reason to think that an observed relation between a polymorphism and a physiological trait such as bone mass would be affected by ethnicity; rather, it may actually explain the ethnic difference in bone mass.

Although much of the literature since Morrison *et al* first reported their VDR results has been negative, it seems that the age ranges of the subjects and the sample sizes of different studies may account for some of the apparently contradictory findings.²⁴ Furthermore, one would expect the relation between bone mass and the VDR to be complex. Several investigators have suggested that there may be linkage of this RFLP with other genes that control bone and mineral metabolism. Such genes may affect intestinal calcium transport¹⁴ or absorption²⁵ or other influences on bone mass. The VDR itself may modify the effect of vitamin D levels on bone turnover,^{26 27} and some studies have indicated that increased calcium intake may modify the effect of the VDR genotype on the rate of bone loss.^{28 29} We suggest that further studies including these other variables and carried out in larger samples of ethnically diverse populations of adolescents and young adults would help to elucidate some of the issues that remain unresolved.

In summary, our data and that of others provide support for the possibility that ethnic differences in VDR genotype frequencies, together with the observed association between the genotypes and bone mass, may partially explain ethnic differences in bone mass. Our data also suggest that this influence may become important during the accumulation of peak bone mass.

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